



**University of
Zurich**^{UZH}

**Zurich Open Repository and
Archive**

University of Zurich
University Library
Strickhofstrasse 39
CH-8057 Zurich
www.zora.uzh.ch

Year: 2015

The influence of stromal cells on the pigmentation of tissue-engineered dermo-epidermal skin grafts

Biedermann, Thomas ; Böttcher-Haberzeth, Sophie ; Klar, Agnieszka S ; Widmer, Daniel S ; Pontiggia, Luca ; Weber, Andreas D ; Weber, Daniel M ; Schiestl, Clemens ; Meuli, Martin ; Reichmann, Ernst

Abstract: It has been shown in vitro that melanocyte proliferation and function in palmoplantar skin is regulated by mesenchymal factors derived from fibroblasts. Here, we investigated in vivo the influence of mesenchymal-epithelial interactions in human tissue-engineered skin substitutes reconstructed from palmar- and non-palmoplantar-derived fibroblasts. Tissue-engineered dermo-epidermal analogs based on collagen type I hydrogels were populated with either human palmar or non-palmoplantar fibroblasts and seeded with human non-palmoplantar-derived melanocytes and keratinocytes. These skin substitutes were transplanted onto full-thickness skin wounds of immuno-incompetent rats. Four weeks after transplantation the development of skin color was measured and grafts were excised and analyzed with regard to epidermal characteristics, in particular melanocyte number and function. Skin substitutes containing palmar-derived fibroblasts in comparison to non-palmoplantar derived fibroblasts showed a) a significantly lighter pigmentation; b) a reduced amount of epidermal melanin granules; and c) a distinct melanosome expression. However, the number of melanocytes in the basal layer remained similar in both transplantation groups. These findings demonstrate that human palmar fibroblasts regulate the function of melanocytes in human pigmented dermo-epidermal skin substitutes after transplantation, whereas the number of melanocytes remains constant. This underscores the influence of site-specific stromal cells and their importance when constructing skin substitutes for clinical application.

DOI: <https://doi.org/10.1089/ten.TEA.2014.0327>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-103044>

Journal Article

Accepted Version

Originally published at:

Biedermann, Thomas; Böttcher-Haberzeth, Sophie; Klar, Agnieszka S; Widmer, Daniel S; Pontiggia, Luca; Weber, Andreas D; Weber, Daniel M; Schiestl, Clemens; Meuli, Martin; Reichmann, Ernst (2015). The influence of stromal cells on the pigmentation of tissue-engineered dermo-epidermal skin grafts. *Tissue Engineering. Part A*, 21(5-6):960-969.

DOI: <https://doi.org/10.1089/ten.TEA.2014.0327>

The influence of stromal cells on the pigmentation of tissue-engineered dermo-epidermal skin grafts

Thomas Biedermann^{1,4*}, Ph.D., Sophie Böttcher-Haberzeth^{1,2,4*}, M.D., Agnieszka S. Klar^{1,4}, M.Sc., Daniel S. Widmer³, Ph.D., Luca Pontiggia^{1,4}, Ph.D., Andreas D. Weber^{2,4}, M.D., Daniel M. Weber^{2,4}, M.D., Clemens Schiestl^{2,4}, M.D., Martin Meuli^{2,4}, M.D., Ernst Reichmann^{1,4}, Ph.D.

(1) University Children's Hospital Zurich, Tissue Biology Research Unit, Zurich, Switzerland

(2) University Children's Hospital Zurich, Department of Surgery, Zurich, Switzerland

(3) University Hospital Zurich, Department of Dermatology, Zurich, Switzerland

(4) Children's Research Center, University Children's Hospital Zurich, Zurich, Switzerland

* Authors contributed equally to this paper

Corresponding author:

Prof. Ernst Reichmann, Ph.D.

Tissue Biology Research Unit

University Children's Hospital Zurich

August Forel Street 7

8008 Zurich

Switzerland

Tel: +41 6348911

Fax: +41 6348918

Email: Ernst.reichmann@kispi.uzh.ch

The work was performed in Zurich, Switzerland.

Abstract

It has been shown *in vitro* that melanocyte proliferation and function in palmoplantar skin is regulated by mesenchymal factors derived from fibroblasts. Here, we investigated *in vivo* the influence of mesenchymal-epithelial interactions in human tissue-engineered skin substitutes reconstructed from palmar- and non-palmoplantar-derived fibroblasts. Tissue-engineered dermo-epidermal analogs based on collagen type I hydrogels were populated with either human palmar or non-palmoplantar fibroblasts and seeded with human non-palmoplantar-derived melanocytes and keratinocytes. These skin substitutes were transplanted onto full-thickness skin wounds of immuno-incompetent rats. Four weeks after transplantation the development of skin color was measured and grafts were excised and analyzed with regard to epidermal characteristics, in particular melanocyte number and function. Skin substitutes containing palmar-derived fibroblasts in comparison to non-palmoplantar derived fibroblasts showed a) a significantly lighter pigmentation; b) a reduced amount of epidermal melanin granules; and c) a distinct melanosome expression. However, the number of melanocytes in the basal layer remained similar in both transplantation groups. These findings demonstrate that human palmar fibroblasts regulate the function of melanocytes in human pigmented dermo-epidermal skin substitutes after transplantation, whereas the number of melanocytes remains constant. This underscores the influence of site-specific stromal cells and their importance when constructing skin substitutes for clinical application.

Keywords: Human pigmented dermo-epidermal skin substitutes – Palmo-plantar fibroblasts – Melanocytes - Skin color – Mesenchymal-epidermal interaction

Introduction

The human palmoplantar epidermis exhibits distinct differences compared to interfollicular epidermis, most importantly with regard to epidermal marker expression and melanocyte function.¹ In this respect, Cytokeratin 9 is a marker expressed exclusively by palmoplantar suprabasal keratinocytes.² It has been shown that its expression pattern is regulated by fibroblasts of the palms and soles through an epithelial-mesenchymal interaction.^{3,4} This influence could be demonstrated when pure non-palmoplantar epidermal sheets were grafted to wounds of the human sole: keratinocytes adapted phenotypical characteristics of palmoplantar skin and expressed the specific marker Cytokeratin 9.⁵

This site-specific epithelial-mesenchymal interaction in palmoplantar skin also has an influence on melanocyte proliferation and differentiation. Yamaguchi et al.⁶ showed *in vitro* that palmoplantar fibroblasts suppressed proliferation and pigmentation of melanocytes through Dickkopf 1 (DKK1), an inhibitor of the Wnt signaling pathway in melanocytes, obviously leading to the lighter skin color of the palms and soles.⁷⁻⁹ This influence was seen when non-palmoplantar split-thickness skin grafts containing non-palmoplantar fibroblasts were transplanted to the sole: The graft remained darker than the surrounding skin. In contrast, non-palmoplantar epidermal sheets containing melanocytes and no fibroblasts, showed a clear hypopigmentation when transplanted to palmoplantar wounds in human patients.¹⁰

Interestingly, opinions differ regarding melanocyte density in palmoplantar human skin. According to Yamaguchi et al., melanocyte density in palmoplantar skin is significantly lower than in interfollicular skin, thereby impacting skin color. In contrast, according to Hasegawa et al., melanocytes exist in a similar density but with a reduced melanogenic activity in palmoplantar skin. This leads to a decreased number of mature melanised melanosomes in melanocytes accounting for the lighter skin color in palmoplantar skin.

Although many components regarding palmoplantar epithelial-mesenchymal interactions have been studied *in vitro* or observed clinically,³⁻⁸ little is known about direct and objective *in vivo* results showing a difference between palmoplantar and non-palmoplantar

fibroblast with respect to their influence on skin pigmentation. Since several years, we are investigating diverse characteristics and properties of dermo-epidermal skin substitutes (DESS) for future clinical application to full-thickness skin defects.¹¹⁻¹⁵ The importance of site-specific, cellular epidermal and dermal components has been shown in the context of reproducing the original donor skin color in our pigmented skin substitutes.¹⁶ Moreover, the applied pre-clinical model enables us to analyze the influence of different fibroblast populations in an *in vivo* setting.

Herein, we investigated the influence of two different human dermal fibroblast populations on pigmentation in human tissue-engineered DESS. We used human palmar and non-palmoplantar fibroblasts in combination with different ratios of non-palmoplantar melanocytes and keratinocytes in DESS for an *in vivo* assay on immuno-incompetent rats. Graft color, pigmentation, and histological characteristics, in particular melanocyte number and function, were analyzed four weeks after transplantation.

Materials and Methods

Human skin samples

The study was performed according to the “Declaration of Helsinki Principles” and after permission by the ethic commission of the Canton Zurich. For the use of foreskins or skin of accessory digits removed in cases of polydactyly parents and/or patients gave informed consent. The age of the children ranged from 1 to 16 years. The foreskins were used for the isolation of keratinocytes, melanocytes, and dermal fibroblasts, the digits for the isolation of dermal fibroblasts from palmar sites. **Foreskins and digits were obtained from different patients.** Tissues samples for histological examinations were prepared for paraffin sections or embedded in OCT compound (Sakura Finetek, Staufien, Germany) and stored at -80 °C.

Isolation and culturing of primary cells

The skin cells were isolated and cultured as described in Böttcher-Haberzeth et al.¹⁷ In brief, small skin pieces (about 3 mm³) **from 3 different dark foreskins (similar L values) and from 3 different digits** were digested overnight in 12 U/ml dispase (BD Biosciences, Allschwil, Switzerland) and Hank's balanced salt solution containing 5 mg/ml gentamycin (all from Invitrogen, Basel, Switzerland) at 4 °C. The epidermis and dermis were subsequently separated by forceps. The epidermis was digested in 1% trypsin and 5 mM EDTA (all from Invitrogen) for 10 minutes at 37 °C. Keratinocytes were harvested and cultured in serum-free keratinocyte medium (SFM) containing 25 mg/ml bovine pituitary extract, 0.2 ng/ml epidermal growth factor, and 5 mg/ml gentamycin (all from Invitrogen). Melanocytes were harvested and cultured in melanocyte growth medium, containing 0.4% bovine pituitary extract, 1 ng/ml basic fibroblast growth factor, 5 mg/ml insulin, 0.5 mg/ml hydrocortison, 10 ng/ml Phorbol Myristate Acetate (all from PromoCell, Heidelberg, Germany), and 5 mg/ml gentamycin (Invitrogen). The dermal tissue was further digested in 2 mg/ml collagenase blend F (Sigma, Buchs, Switzerland) for 4 hours at 37 °C. Dermal fibroblasts were grown in DMEM containing 10% fetal calf serum (FCS), 4 mM L-alanyl-L-glutamine, 1 mM sodium pyruvate, and 5 mg/ml gentamycin (all from Invitrogen).

Preparation of dermo-epidermal skin substitutes

The human dermo-epidermal skin substitutes (DESS) were prepared as described by Braziulis et al.¹³ Briefly, **3 ml of bovine collagen type I** (5 mg/mL; Symatase Biomateriaux, Chaponost, France; containing Neutral Red pH indicator) was mixed with 1×10^5 human fibroblasts (from palmar skins or from foreskins, passage 1-2) and chilled neutralization buffer containing 0.5 M NaOH (Sigma), **and casted into 6-well cell culture inserts** (3.0 μ m pore-size) (BD Biosciences). Dermal equivalents were grown in DMEM containing 10% fetal calf serum (FCS), 4 mM L-alanyl-L-glutamine, 1 mM sodium pyruvate, and 5 mg/ml gentamycin for 6 days. On each dermal equivalent 5×10^5 keratinocytes (passage 1-2) and melanocytes (passage 1-2) isolated and cultured from foreskins were seeded in a ratio of melanocytes:keratinocytes 1:5 or 1:10. Skin analogs were prepared with palmar (n=9, **with 1:5 melanocytes:keratinocytes**), palmar (n=9, **with 1:10 melanocytes:keratinocytes**) and non-palmar (n=9, **with 1:5 melanocytes:keratinocytes**) fibroblast **(from the 9 skin analogs with different seeding ratios three analogs were prepared from each of the three different donors)**. DESS were cultured for one week in a 5:1 mix of keratinocyte medium and melanocyte growth medium (Promocell, Heidelberg, Germany), and subsequently transplanted.

Transplantation of DESS

The surgical protocol was approved by the local Committee for Experimental Animal Research (permission number 115/2012). Immuno-incompetent female nu/nu rats, 8–10 weeks old (Harlan Laboratories, Netherlands), were prepared and anesthetized as described by Schneider et al.¹⁸ Skin analogs (containing palmar (n=18) and non-palmar (n=9) fibroblast) were transplanted onto full-thickness skin defects created surgically on the backs of animals. Wound closure from surrounding rat skin was prevented and the skin substitutes were protected by surgical steel rings (26 mm in diameter) sutured to the skin defects using nonabsorbable polyester sutures (Ethibond®, Ethicon, Zug, Zurich). DESS were covered with a silicone foil (Silon-SES, BMS, Allentown, USA), a polyurethane sponge (Ligasano,

Ligamed, Ötztal, Austria), and a tape as wound dressing. Dressing changes were done once per week. After 4 weeks photographic documentations and chromameter measurements were performed, the transplanted skin substitutes were excised in toto and processed for cryo- and paraffin sections and transmission electron microscopy.

Chromameter measurements

In 1976, the color vision is described as trichromatic with uniform, three-dimensional color space ($L^*a^*b^*$) by the International Commission on Illumination (Commission internationale de l'Eclairage, CIE). In the color space, the L value correlates to perceived lightness. It ranges from absolute black (0) to absolute white (+100). The visible spectrum of light reflected from foreskins and skin analogs was measured, and a color change was recorded by the L-value (mean \pm SD) as it is the most sensitive of the trichromatic values of the reflectance spectroscopy. **For the statistical significance test an unpaired two-sided student's t-test was performed.** The spectroscopy was conducted by employing a Chromameter CR-200 (Minolta, Osaka, Japan).

Histological analysis

Paraffin sections (5 μ m) were stained with hematoxylin and eosin (Sigma) to assess the histological morphology. The Fontana Masson technique was performed to visualize melanin.

Immunohistochemical staining

Immunofluorescence staining was performed as described in Pontiggia et al.¹¹: The following antibodies were used for immunofluorescence: HMB45 (1:50), E-cadherin (clone NCH-38, 1:30), all from Dako, Baar, Switzerland; laminin-5 (clone P3H9-2, 1:100), Tyr (M-19, polyclonal, 1:50), Tyrp-1 (H-90, polyclonal, 1:50) all from Santa Cruz, Heidelberg, Germany; CK1 (clone LHK1, 1:200, Chemicon, Zug, Switzerland); CD90/Thy-1 (clone AS02, 1:100, Calbiochem, Dietikon, Switzerland). Photos of immunofluorescence stainings were taken

with a DXM1200F digital camera connected to a Nikon Eclipse TE2000-U inverted microscope. The device is equipped with Hoechst 33342-, FITC-, and TRITC-filter sets (Nikon AG, Switzerland; Software: Nikon ACT-1 vers. 2.70). Images were processed with Photoshop 10.0 (Adobe Systems Inc., Basel, Switzerland).

Immunohistochemical analysis was performed as described before.¹⁹ Tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were deparaffinized in xylene and rehydrated. For antigen retrieval, slides were heated with cell conditioner 1 (Ventana Medical Systems, Tucson, AZ). Sections were incubated with MITF antibody (clone 5pD5, 1:50, Abcam, Cambridge, UK). The antibody binding was visualized using the detection kit by Ventana (iVIEW DAB detection kit (Ventana)). Slides were counterstained with hematoxylin.

Electron microscopy

Samples (approximately 1 mm³) were prefixed for two hours in 0.1 M cacodylate buffer (Merck, Darmstadt, Germany), pH 7.3 containing 2.5% glutaraldehyde and washed in cacodylate buffer for transmission electron microscopy analysis. Tissue blocs were postfixed for one hour with an aqueous solution of 1% OsO₄ and 1.5% K₄Fe(CN)₆, dehydrated, and finally embedded in EPON 812 (Promega AG, Dübendorf, Switzerland). Ultrathin sections (50–70 nm) were collected on copper grids, contrasted with 4% uranyl acetate, and 3% lead citrate. Examination of sections was performed with a CM 100 transmission electron microscope (Philips, Zurich, Switzerland). All reagents were from Sigma unless mentioned otherwise.

Statistical analysis

Melanocytes in the basal layer of the skin analogs were evaluated by counting MITF positive cells four times in each of five representative sections from the skin samples. The results were calculated to display the mean ± SD graphically (Microsoft Excel).

Results

Human palmar skin shows distinctive macroscopic and microscopic characteristics

Macroscopically, color discrepancy between palmar (as well as plantar) and interfollicular skin are known, although, it is more obvious in skin type V-VI (Fig 1a) than in skin type I-II (Fig 1b) according to the Fitzpatrick scale of skin type pigmentation.

Microscopically, palmar skin shows several unique characteristics: a very thick stratum corneum (Fig 1 c, white two-sided arrow), a distinctive stratum lucidum, no hair follicles, but abundant eccrine sweat glands and pronounced rete ridges (Fig 1c, black arrows). Highlighting melanocytes with an HMB45 antibody, sporadic melanocytes contacting the basement membrane (stained with an anti-Laminin 5 antibody) can be seen (Fig 1d). Regarding epidermal marker expression, Cytokeratin 9 (CK9), expressed exclusively by palmoplantar suprabasal keratinocytes, is superimposed with CK1 (Fig 1e). As in interfollicular skin, all epidermal cells express E-Cadherin and dermal fibroblasts are positive for a CD90 staining (Fig 1f).

Pigmentation of human DESS prepared with human dermal fibroblasts from different body sites

To easily survey and analyze the influence of fibroblasts on the pigmentary system, DESS were produced with keratinocytes and melanocytes from dark non-palmoplantar skin in the epidermal compartment. The dermal compartment was produced either with human palmar fibroblasts or with human dermal fibroblasts from the same dark skin biopsy as the epidermal cells. Four weeks after transplantation onto immuno-incompetent rats, there was a clear-cut color difference between the two groups of grafts (Fig 2a, b). When fibroblasts from palmar skin were used, the skin substitutes showed a very light color (Fig 2a). In contrast, the substitutes with the same epidermal cells but produced with dermal fibroblasts from non-palmoplantar skin showed a dark skin similar to the color of the donor biopsy.

These results could be confirmed by objective color measurements using a chromameter (Fig 2c). Transplants with all three cell types (keratinocytes, melanocytes,

fibroblasts) from the dark non-palmar skins ($n=9$, $L=43.4$, $SD: 0.98$) showed the same measurements as the donor skin biopsies ($L=44.8$, $SD: 0.82$). However, measurements from the transplants constructed with palmar fibroblasts differed significantly ($p<0.0001$) from transplants with non-palmar fibroblasts or from the original dark foreskins.

To additionally investigate a possible influence of various epidermal cell seeding ratios, the palmar fibroblast-derived DESS were transplanted with different initial melanocyte:keratinocyte seeding ratio of 1:5 ($n=9$) and 1:10 ($n=9$). The L values obtained were similar, namely with a ratio of 1:5 the L value was 61.5 ($SD: 0.51$), with a ratio of 1:10 the L value was 58.0 ($SD: 3.29$). Hence, the different seeding number of melanocytes did not influence skin color.

Histologically, all transplants showed the development of a similar stratified and cornified epidermis four weeks after transplantation (Fig 3a, b, **higher magnifications thereof in c, d**). No stratum lucidum or thick stratum corneum could be detected in the DESS produced with palmar fibroblasts.

Melanin distribution in the transplanted DESS was revealed by a Fontana Masson staining. All transplants showed melanin throughout their entire epidermis (Fig **3e-h**), located mainly as supranuclear caps in the keratinocytes (arrows, Fig **3g, h**). However, a clear difference regarding melanin quantity between transplants with palmar (Fig **3e, g**) and non-palmar fibroblasts (Fig **3f, h**) was evident. Substitutes produced with non-palmar fibroblasts showed a higher concentration of melanin in the basal layer (keratinocytes and melanocytes) as well as in suprabasal keratinocytes.

To demonstrate melanin uptake by keratinocytes and physiological melanocyte position, transmission electron microscopy was performed on DESS produced with palmar fibroblasts. Melanosome-containing melanocytes were detected in the basal layer of the epidermis (M in Fig 4a) with dendrites reaching into suprabasal layers (Fig 4a, black arrow head). Adjacent keratinocytes (K in Fig 4a), affirmed by their possession of desmosomes

(Fig 4a, white arrows), showed melanin inclusions in a physiological supranuclear position (Fig 4a, black arrows).

Transmission electron microscopy photographs were used to determine the number of melanocytes in the basal layer of all DESS (Fig 4b). Thereby, a similar percentage of melanocytes in the basal layer in the different groups was revealed: 20.2% (± 3.89) in DESS with palmar fibroblasts and a seeded melanocyte:keratinocyte ratio of 1:5; 19.7% (± 4.85) in DESS with palmar fibroblasts and a seeded melanocyte:keratinocyte ratio of 1:10. The different initial seeding ratio of melanocytes:keratinocytes (1:5 or 1:10) *in vitro* did not have an influence on the final melanocyte:keratinocytes ratio of about 1:5 observed after 4 weeks *in vivo*.

DESS with non-palmar fibroblasts and a seeded melanocyte:keratinocyte ratio of 1:5 displayed a percentage of 23.6% (± 1.91) melanocytes in the basal layer of the epidermis.

In addition, a quantification of microphthalmia-associated transcription factor (MITF)-positive melanocytes using immunohistochemistry stainings resulted in the same percentages of basal melanocytes in all transplants (Supplementary Fig 1a, b, **higher magnifications in c, d**).

Skin substitutes produced with either palmar or nonpalmar fibroblasts differ in melanosome maturation

Melanosome maturation in human skin differs depending on body site and skin pigmentation type. Melanocytes of palmar skin produce early melanosome stages I-II (shown in Fig. 5 by an HMB45 staining), whereas stages III-IV (shown by a Tyrp-1 staining) are not produced (Fig 5a, b, c). Non-palmoplantar dark pigmented skin express more mature melanosome stages III-IV (Fig 5d, e, f). However, non-palmoplantar light pigmented skin shows predominantly early stages I-II (Fig 5g, h, i). Our DESS constructed with palmar fibroblasts were only positive for HMB45 (stages I-II) (Fig 5j, k, l), in accordance to the expression pattern of palmar skin (Fig 5a, b, c). In contrast, DESS constructed with non-palmar fibroblasts showed expression of both, HMB45 and Tyrp-1 (Fig 5m, n, o). (**Higher**

magnifications for HMB45 and Tyrp-1 expression pattern in palmar and non-palmar DESS are shown in Supplementary Figure 1 e-h.)

In addition, we investigated the expression of tyrosinase (Tyr) regarding melanosome maturation (Supplementary Figure 2). **In normal human skin, palmar melanocytes produce no tyrosinase, whereas non-palmoplantar melanocytes express tyrosinase (data not shown). Our DESS, constructed with palmar fibroblasts, were only positive for HMB45 (Supplementary Fig 2a-c, higher magnifications in d and e), but not positive for tyrosinase expression, again in accordance to the expression pattern of palmar skin. Moreover, DESS constructed with non-palmar fibroblasts showed expression of tyrosinase (Supplementary Fig 2f-h, higher magnifications in i and j).**

Skin substitutes produced with either palmar or non-palmar fibroblasts differ in cytokeratin 9 expression

To analyze the palmoplantar specific marker CK9 in non-palmoplantar keratinocytes of our DESS, immunofluorescence staining using an anti-CK9 antibody was performed. Skin substitutes produced with palmar fibroblasts revealed a scattered CK9 expression pattern in suprabasal keratinocytes, whereas CK1 was evenly expressed in all suprabasal cells (Supplementary Fig 3a). In contrast, the same non-palmoplantar keratinocytes in DESS produced with non-palmoplantar fibroblasts were negative for CK9 in keratinocytes, while CK1 was expressed by all suprabasal keratinocytes (Supplementary Fig 3b).

To prove the persistence of human fibroblasts four weeks after transplantation, skin substitutes were stained with an anti-CD90 staining, detecting specifically human fibroblasts. In both groups, abundant human fibroblasts were present underneath the human E-cadherin positive epidermis (Supplementary Fig 3c, d).

Discussion

Structural differences between human palmoplantar and interfollicular skin, in particular the influence of the mesenchyme on epidermal phenotype development and expression pattern of different cytokeratins are known and have been studied previously.^{5,10} It has also been demonstrated that by transplanting interfollicular skin onto the palms or soles, the original phenotype of the epidermis remains as long as fibroblasts from the initial site are included.^{3,4} Yet, little is known about the specific influence of palmoplantar or interfollicular fibroblasts on melanocyte number and function in an *in vivo* model. To further investigate the hypothesis that palmoplantar fibroblasts act on keratinocytes and melanocytes, i.e. specifically on melanogenesis, we compared pigmented DESS constructed with human palmar respectively non-palmoplantar fibroblasts in an *in vivo* assay. Generally, we could show here in an *in vivo* assay that human palmar fibroblasts regulate the melanogenesis of melanocytes in human pigmented DESS after transplantation whereas the number of melanocytes was identical in all transplants.

Macroscopically, we could detect a striking and significant difference in skin color when palmar and non-palmar fibroblasts were used in the otherwise same skin substitute. This difference was examined in more detail. **In accordance with previously described findings about the physiological distribution of melanocytes in the basal layer of the human epidermis, we chose a initial seeding ratio of melanocytes to keratinocytes in our DESS of 1:5.^{1,20,21} In addition, we also prepared DESS *in vitro* with a melanocyte to keratinocyte ratio of 1:10 to investigate the influence of palmar fibroblasts on the proliferation of melanocytes. Regarding the number of melanocytes *in vivo*, we found a consistent ratio of about 1:5 of melanocytes to keratinocytes in the basal layer of the epidermis in all DESS 4 weeks after transplantation despite the fact that we used both a 1:5 and a 1:10 ratio of melanocytes to keratinocytes.**

This is an important finding, as opinions regarding the influence of palmoplantar fibroblasts on melanocyte number are controversial. Yamaguchi et al.⁴ claim that there are less melanocytes in palmoplantar skin than in interfollicular skin and that palmar fibroblasts

have a suppressive effect on melanocyte proliferation *in vitro*.⁶ However, Hasegawa et al.²² describe that there are equal numbers of melanocytes in palmoplantar and interfollicular skin. Here, in contrast to Yamaguchi et al.⁶, we did not observe an inhibitory influence of palmar fibroblasts on melanocyte proliferation in our DESS, as the equal physiological melanocyte to keratinocytes ratio was established. Obviously, in terms of melanocyte number regulation, keratinocytes display a greater influence than fibroblasts as also suggested by Scott and Haake.²² **Moreover, we had already used melanocytes and keratinocytes in different ratios (1:1, 1:5, and 1:10) with non-palmoplantar fibroblasts in DESS.¹⁶ There, we also consistently detected a ratio of 1:5 (melanocytes:keratinocytes) after transplantation, indicating the establishment of a physiologically 1:5 melanocyte:keratinocyte ratio under homeostatic conditions.**

With regard to melanogenic activity, we could show that the epidermal melanin content in the basal and suprabasal keratinocyte layers was significantly lower in skin substitutes constructed with palmar fibroblasts, resulting in higher chromameter values. This could be confirmed by the presence of less melanin granules shown by Fontana Masson staining, as well as by TEM analysis. More specifically, we detected a reduced formation of mature melanosomes revealed by an absent expression of Tyrp-1, a marker for mature melanosome stages III-IV. These results are in accordance with previous studies showing a suppression of melanosome maturation *in vitro* as well as in normal palmoplantar skin.^{6,23}

These results indicate that we can reproduce mesenchymal-epithelial interactions with palmar and non-palmar fibroblasts acting on the pigmentary system of a non-palmoplantar epidermis in our transplantation model. We suggest that palmar fibroblasts act only on melanosome maturation and on melanin transfer to keratinocytes. That is to say, melanosome maturation in melanocytes is suppressed and the transfer of melanin from melanocytes to keratinocytes is decreased. We speculate that this influence could be caused by DKK1, an inhibitor of the canonical Wnt signaling pathway, shown to be expressed in higher amounts by palmoplantar fibroblasts.⁶

Although the epidermis of skin substitutes produced with palmar fibroblasts did not show an extremely thick stratum corneum or a stratum lucidum, keratinocytes started to express CK9, an exclusive differentiation marker of palmoplantar skin. This is somewhat surprising, as keratinocytes used in skin substitutes were derived from non-palmoplantar skin, and CK9 expression was previously considered to be regulated endogenously.⁵ On the other hand, several studies have demonstrated that mesenchymal factors can modulate the phenotype of epidermal keratinocytes.^{3,24-28}

In conclusion, this study demonstrates a specific influence of human palmoplantar fibroblasts on the pigmentation of non-palmoplantar epidermis in DESS transplanted in an animal model. It emphasizes the importance of using site-specific stromal cells when constructing pigmented dermo-epidermal skin grafts for clinical application, especially when aiming to produce skin grafts matching the color of the site to be transplanted.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgments

We thank Gery Barmettler (Center for Microscopy and Image Analysis, University Zurich) and Ines Kleiber-Schaaf (Department of Dermatology, University Hospital, Zurich) for precious help in the preparation and analysis of the probes for transmission electron microscopy and histology. This work was financially supported by the EU-FP7 project EuroSkinGraft (FP7/2007-2013: grant agreement n° 279024), by the EU-FP7 (MultiTERM, grant agreement n° 238551) and the Clinical Research Priority Programs (KFSP: From basic research to the clinic: Novel tissue engineered skin grafts for Zurich) of the Faculty of Medicine of the University of Zurich. We are particularly grateful to the Fondation Gaydoul and the sponsors of “DonaTissue” (Thérèse Meier and Robert Zingg) for their generous financial support and interest in our work.

References

1. Szabo G. The number of melanocytes in human epidermis. *Br Med J.* **1**, 1016, 1954
2. Knapp AC, Franke WW, Heid H, Hatzfeld M, Jorcano JL, and Moll R. Cytokeratin No. 9, an epidermal type I keratin characteristic of a special program of keratinocyte differentiation displaying body site specificity. *J Cell Biol* **103**, 657, 1986.
3. Compton CC, Nadire KB, Regauer S, Simon M, Warland G, O'Connor NE, Gallico GG, and Landry DB. Cultured human sole-derived keratinocyte grafts re-express site-specific differentiation after transplantation. *Differentiation* **64**, 45, 1998.
4. Yamaguchi Y, Kubo T, Tarutani M, Sano S, Asada H, Kakibuchi M, Hosokawa K, Itami S, and Yoshikawa K. Epithelial-mesenchymal interactions in wounds: treatment of palmoplantar wounds by nonpalmoplantar pure epidermal sheet grafts. *Arch Dermatol* **137**, 621, 2001.
5. Yamaguchi Y, Itami S, Tarutani M, Hosokawa K, Miura H, and Yoshikawa K. Regulation of keratin 9 in nonpalmoplantar keratinocytes by palmoplantar fibroblasts through epithelial-mesenchymal interactions. *J Invest Dermatol* **112**, 483, 1999.
6. Yamaguchi Y, Itami S, Watabe H, Yasumoto K, Abdel-Malek ZA, Kubo T, Rouzaud F, Tanemura A, Yoshikawa K, and Hearing VJ. Mesenchymal-epithelial interactions in the skin: increased expression of dickkopf1 by palmoplantar fibroblasts inhibits melanocyte growth and differentiation. *J Cell Biol* **165**, 275, 2004.
7. Yamaguchi Y, Passeron T, Watabe H, Yasumoto K, Rouzaud F, Hoashi T, and Hearing VJ. The effects of dickkopf 1 on gene expression and Wnt signaling by melanocytes: mechanisms underlying its suppression of melanocyte function and proliferation. *J Invest Dermatol* **127**, 1217, 2007.
8. Yamaguchi Y, Passeron T, Hoashi T, Watabe H, Rouzaud F, Yasumoto K, Hara T, Tohyama C, Katayama I, Miki T, and Hearing VJ. Dickkopf 1 (DKK1) regulates skin

pigmentation and thickness by affecting Wnt/beta-catenin signaling in keratinocytes. *FASEB J* **22**, 1009, 2008.

9. Yamaguchi Y, Morita A, Maeda A, and Hearing VJ. Regulation of skin pigmentation and thickness by Dickkopf 1 (DKK1). *J Invest Dermatol Symp Proc* **14**, 73, 2009.

10. Yamaguchi Y, and Yoshikawa K. Cutaneous wound healing: an update. *J Dermatol* **28**, 521, 2001.

11. Pontiggia L, Biedermann T, Meuli M, Widmer D, Böttcher-Haberzeth S, Schiestl C, Schneider J, Braziulis E, Montaña I, Meuli-Simmen C, and Reichmann E. Markers to evaluate the quality and self-renewing potential of engineered human skin substitutes in vitro and after transplantation. *J Invest Dermatol* **29**, 480, 2009.

12. Biedermann T, Pontiggia L, Böttcher-Haberzeth S, Braziulis E, Schiestl C, Meuli M, and Reichmann E. Human eccrine sweat gland cells can reconstitute a stratified epidermis. *J Invest Dermatol* **130**, 1996, 2010.

13. Braziulis E, Diezi M, Biedermann T, Pontiggia L, Schmucki M, Hartmann-Fritsch F, Luginbühl J, Schiestl C, Meuli M, and Reichmann E. Modified plastic compression of collagen hydrogels provides an ideal matrix for clinically applicable skin substitutes. *Tissue Eng Part C Methods* **18**, 464, 2012.

14. Klar AS, Böttcher-Haberzeth S, Biedermann T, Schiestl C, Reichmann E, and Meuli M. Analysis of blood and lymph vascularization patterns in tissue-engineered human dermo-epidermal skin analogs of different pigmentation. *Pediatr Surg Int* **30**, 223, 2014.

15. Hartmann-Fritsch F, Biedermann T, Braziulis E, Luginbühl J, Pontiggia L, Böttcher-Haberzeth S, van Kuppevelt, Faraj KA, Schiestl C, Meuli M, and Reichmann E. Collagen hydrogels strengthened by biodegradable meshes are a basis for dermo-epidermal skin

grafts intended to reconstitute human skin in a one-step surgical intervention. *J Tissue Eng Regen Med* advance online publication, doi: 10.1002/term.1665

16. Böttcher-Haberzeth S, Klar AS, Biedermann T, Schiestl C, Meuli-Simmen C, Reichmann E, and Meuli M. Trooping the colour - restoring the original donor skin colour by addition of melanocytes to skin substitutes. *Pediatr Surg Int* **29**, 239, 2013.

17. Böttcher-Haberzeth S, Biedermann T, Pontiggia L, Braziulis E, Schiestl C, Hendriks B, Eichhoff OM, Widmer DS, Meuli-Simmen C, Meuli M, and Reichmann E. Human eccrine sweat gland cells turn into melanin-uptaking keratinocytes in stratifying dermo-epidermal skin substitutes. *J Invest Dermatol* **133**, 316, 2013.

18. Schneider J, Biedermann T, Widmer D, Montaña I, Meuli M, Reichmann E, and Schiestl C. Matriderm versus Integra: a comparative experimental study. *Burns* **35**, 51, 2009.

19. Kiowski G, Biedermann T, Widmer DS, Civenni G, Burger C, Dummer R, Sommer L, and Reichmann E. Engineering melanoma progression in a humanized environment in vivo. *J Invest Dermatol* **132**, 144, 2012.

20. Fitzpatrick TB, and Breathnach AS. The epidermal melanin unit system. *Dermatol Wochenschr* **147**, 481, 1963.

21. Frenk E, and Schellhorn JP. Morphology of the epidermal melanin unit. *Dermatologica* **139**, 271, 1969.

22. Scott GA, and Haake AR. Keratinocytes regulate melanocyte number in human fetal and neonatal skin equivalents. *J Invest Dermatol* **97**, 776, 1991.

23. Hasegawa J, Goto Y, Murata H, Takata M, Saida T, and Imokawa G. Downregulated melanogenic paracrine cytokine linkages in hypopigmented palmoplantar skin. *Pigment Cell Melanoma Res* **21**, 687, 2008.

24. Yamaguchi Y, Hearing VJ, Itami S, Yoshikawa K, and Katayama I. Mesenchymal-epithelial interactions in the skin: aiming for site-specific tissue regeneration. *J Dermatol Sci* **40**, 1, 2005.
25. Hedley SJ, Layton C, Heaton M, Chakrabarty KH, Dawson RA, Gawkrödger DJ, and MacNeil S. Fibroblasts play a regulatory role in the control of pigmentation in reconstructed human skin from skin types I and II. *Pigment Cell Res* **15**, 49, 2002.
26. Okazaki M, Yoshimura K, Fujiwara H, Suzuki Y, and Harii K. Induction of hard keratin expression in non-nail-matrix keratinocytes by nail-matrix fibroblasts through epithelial-mesenchymal interactions. *Plast Reconstr Surg* **111**, 286, 2003
27. Okazaki M, Yoshimura K, Suzuki Y, Suzuki Y, and Harii K. Effects of subepithelial fibroblasts on epithelial differentiation in human skin and oral mucosa: heterotypically recombined organotypic culture model. *Plast Reconstr Surg* **112**, 784, 2003.
28. Choi W, Wolber R, Gerwat W, Mann T, Batzer J, Smuda C, Liu H, Kolbe L, and Hearing VJ. The fibroblast-derived paracrine factor neuregulin-1 has a novel role in regulating the constitutive color and melanocyte function in human skin. *J Cell Sci* **123**, 3102, 2010.

Reprint author:

Prof. Ernst Reichmann, Ph.D.

Tissue Biology Research Unit

University Children's Hospital Zurich

August Forel Street 7

8008 Zurich

Switzerland

Tel: +41 6348911

Fax: +41 6348918

Email: Ernst.reichmann@kispi.uzh.ch

Figure Legends

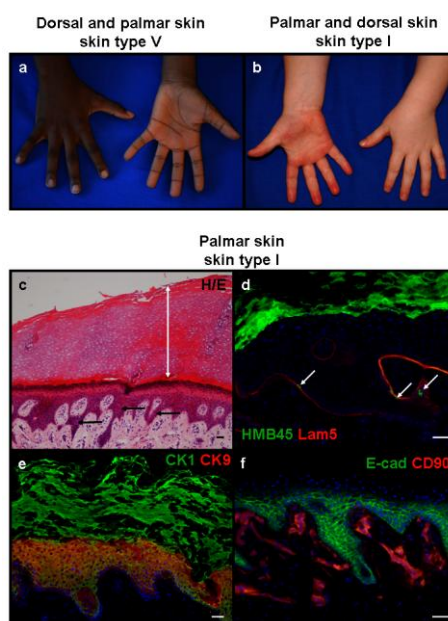


Figure 1. Palmar skin of children of two different skin types and histological appearance of palmar skin. (a) Palmar and dorsal skin of Fitzpatrick skin type V displaying the hypopigmented palmar skin compared to the dark pigmented dorsal skin. **(b)** Palmar and dorsal skin of Fitzpatrick skin type I displaying no subjective difference in pigmentation between the palmar and dorsal side. **(c)** Hematoxylin/Eosin staining of Fitzpatrick skin type I palmar skin. Note the thick stratum corneum (white two-sided arrow), pronounced rete ridges (black arrows) and the absence of hair follicles. **(d)** HMB45 positive melanocytes (green, white arrows) in the basal layer of palmar skin. Laminin 5 (Lam5, red) represents a marker for the basement membrane. **(e)** Cytokeratin 1 (green) and Cytokeratin 9 (red) are expressed in suprabasal keratinocytes in palmar skin in an overlapping manner. **(f)** E-cadherin (green) expression of keratinocytes and CD90 (red) expression in dermal cells of palmar skin. Counterstaining (d-f) for cell nuclei (blue) with Hoechst 33342. Bars (c-f) = 50 μ m.

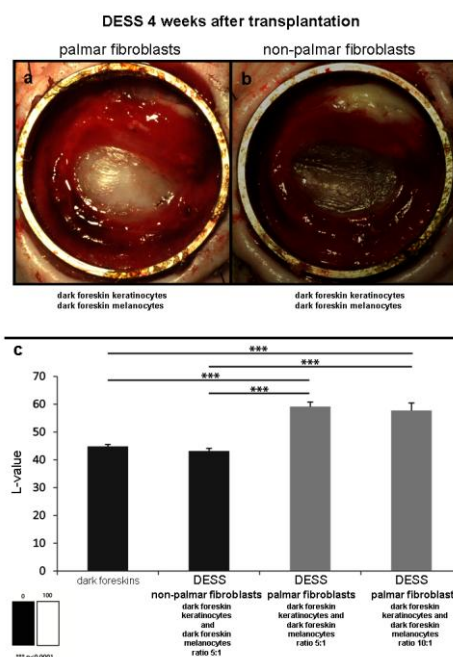


Figure 2. Macroscopic appearance of transplanted skin analogs and chromameter measurements. (a, b) DESS containing dark foreskin-derived keratinocytes, melanocytes, and palmar-derived fibroblasts (a), or non-palmar-derived fibroblasts (b), 4 weeks after transplantation into created full-thickness skin wounds on immuno-incompetent rats (melanocyte to keratinocyte ratio in DESS 1:5). (c) Chromameter measurements of dark foreskins (as control), DESS containing non-palmar-derived fibroblasts and DESS (melanocyte to keratinocyte ratio 1:5 or 1:10) containing palmar-derived fibroblasts 4 weeks after transplantation. The L-value represents a range between 0 (absolute black) and 100 (absolute white). Five measurements for 9 different non-palmar and palmar containing fibroblast DESS were performed. Error bars represent mean \pm SD. The rings in (a) and (b) are 2.6 cm in diameter.

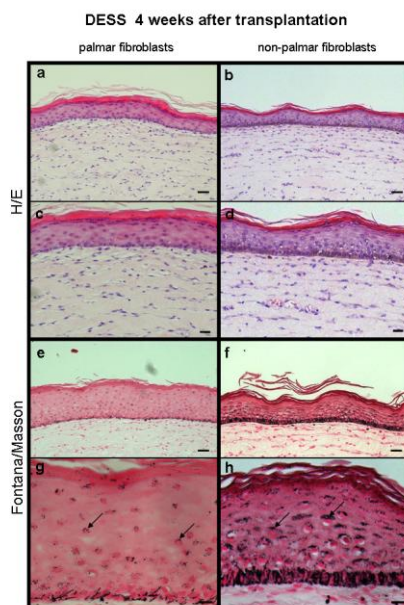


Figure 3. Histological appearance of transplanted skin substitutes 4 weeks after transplantation. (a, b) Hematoxylin/Eosin (H/E) staining of DESS containing palmar-derived fibroblasts (a) and non-palmar-derived fibroblasts (b) reveals a stratified, cornified multilayered epidermis in both types of substitutes. Of note, no thickened stratum corneum could be seen in skin analogs containing palmar fibroblasts. (c, d) Higher magnifications of the H/E staining (a) and (b). (e, f) Fontana-Masson staining of skin analogs containing palmar-derived fibroblasts (e) and non-palmar-derived fibroblasts (f) displays cytoplasmic melanin in cells of the epidermis in both types of skin substitutes. (g, h) Higher magnification of the Fontana-Masson staining. Less melanin is visible in skin analogs with palmar fibroblasts (g) compared to non-palmar fibroblast analogs (h). Melanin stored in supranuclear caps (arrows). Bars (a, b, e, f) = 50 μ m, bars (c, d, g, h) = 25 μ m.

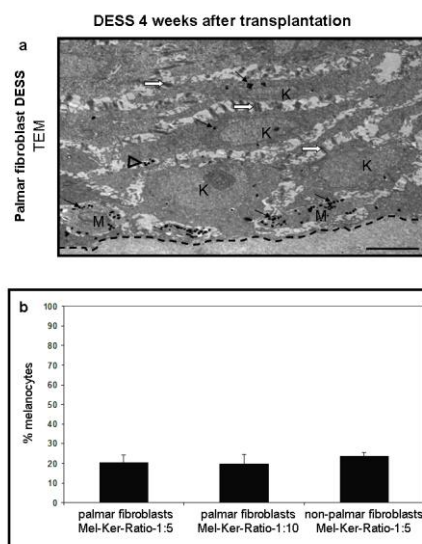


Figure 4. Transmission electron microscopy analysis of transplanted skin substitutes 4 weeks after transplantation. (a) TEM of DESS containing palmar-derived fibroblasts highlights the presence of melanin granules (black arrows) in melanocytes (M) in the basal layer and in keratinocytes (K) in suprabasal layers. The black arrowhead marks a melanocyte dendrite projecting to suprabasal layers. White arrows denote desmosomes of keratinocytes. **Black dashed line** demarcates the dermo-epidermal junction. **(b)** The bars in the diagram represent the percentage of positive melanocytes in the basal layer for skin substitutes containing palmar-derived fibroblasts and non-palmar-derived fibroblasts. Melanocytes are present in the physiological number of about 20% of all basal cells in all types of DESS (Mel-Ker-Ratio = seeded melanocyte to keratinocyte ratio). The bars display the mean \pm SD. Bar (a) = 5 μ m.

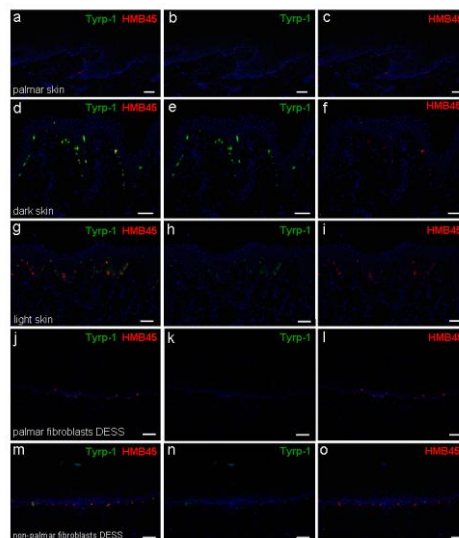
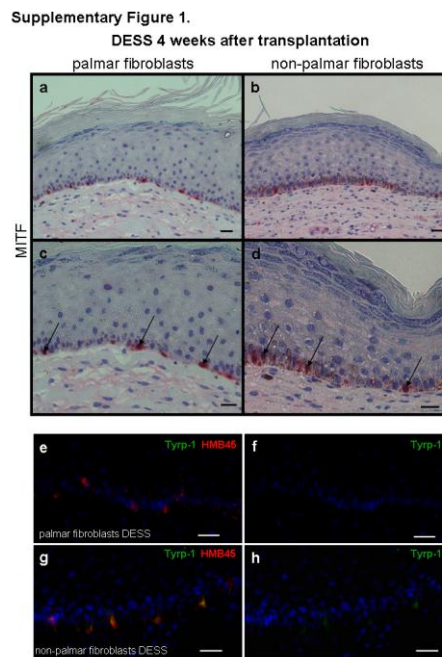
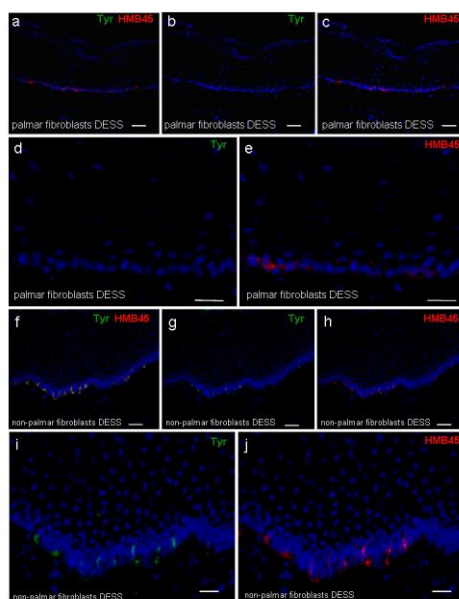


Figure 5. Expression pattern of Tyrp-1 and HMB45 in melanocytes. (a-c) Example of Tyrp-1 (green) and HMB45 (red) expression in melanocytes of human palmar skin. No expression of Tyrp-1 is present. (d-i) Expression of Tyrp-1 (green) and HMB45 (red) in dark pigmented foreskin (d-f) and in light pigmented foreskin (g-i). Note the different presence of Tyrp-1 in (e) and (h). (j-o) Expression pattern of Tyrp-1 (green) and HMB45 (red) in DESS containing palmar-derived fibroblasts (j-l) and non-palmar-derived fibroblasts (m-o). No Tyrp-1 expression is detected in (k), resembling the pattern in (b). Counterstaining (a-o) for cell nuclei (blue) with Hoechst 33342. Bars (a-o) = 50 μ m.



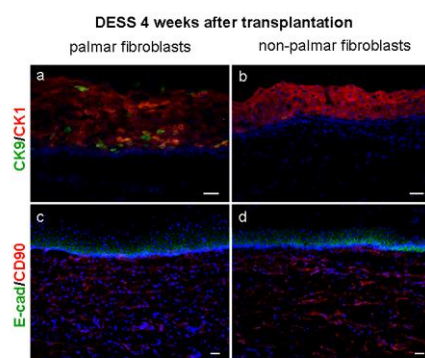
Supplementary Figure 1. Immunohistochemistry staining for MITF in DESS 4 weeks after transplantation and higher magnifications of Tyrp-1 and HMB45 expression pattern. (a, b, c, d) MITF positive melanocytes in skin substitutes containing palmar-derived fibroblasts (a, c) and non-palmar-derived fibroblasts (b, d) are (cell nuclei in red) situated in the basal layer of the epidermis. (c, d) Higher magnifications of a and b, black arrows indicate MITF positive cell nuclei of melanocytes. (e, f, g, h) Expression pattern of Tyrp-1 (green) and HMB45 (red) in DESS containing palmar-derived fibroblasts (e, f) and non-palmar-derived fibroblasts (g, h) (higher magnifications of Figure 5 (j-o)). Bars (a, b) = 50 μ m, (c-h) = 25 μ m.

Supplementary Figure 2.



Supplementary Figure 2. Expression pattern of Tyrosinase and HMB45 in melanocytes. (a-c) Expression pattern of Tyr (green) and HMB45 (red) in DESS containing palmar-derived fibroblasts. No Tyr expression is detected in (b). (d, e) Higher magnifications of b and c. (f-h) Expression pattern of Tyr (green) and HMB45 (red) in DESS containing non-palmar-derived fibroblasts. (i, j) Higher magnifications of g and h. Counterstaining (a-j) for cell nuclei (blue) with Hoechst 33342. Bars (a-c, f-h) = 50 μ m, (d, e, i, j) = 25 μ m.

Supplementary Figure 3.



Supplementary Figure 3. Marker expression in skin substitutes 4 weeks after transplantation. (a, b) Cytokeratin 1 (red) is expressed in all suprabasal cells in DESS with palmar fibroblasts (a) and non-palmar fibroblasts (b). Cytokeratin 9 (green) is present in several suprabasal keratinocytes in skin substitutes containing palmar-derived fibroblasts (a), whereas it is absent in DESS containing non-palmar-derived fibroblasts (b). (c, d) Human CD90 positive fibroblasts (red) are present in the dermal part of the skin substitutes underneath the human epidermal E-cadherin (red) positive keratinocytes in both types of DESS. Counterstaining (a-d) for cell nuclei (blue) with Hoechst 33342. Bars (a-d) = 50 μ m.